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The Epstein-Barr Virus EBNA-2 Gene in Oral Hairy Leukoplakia: Strain Variation, Genetic Recombination, and Transcriptional Expression

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Oral hairy leukoplakia (HLP) lesions frequently contain defective Epstein-Barr virus (EBV) genomes with deletions in the EBNA-2 gene that abundantly replicate and persist within the lesion. To characterize these viral strains and recombinant variants, the EBNA-2 gene in EBV DNA from several different HLP biopsy specimens was analyzed. Amplification of EBNA-2 coding sequences by PCR demonstrated the presence in HLP of intact EBNA-2 genes as well as a variety of internally deleted variants of both EBNA-2A and EBNA-2B. Some of the deletion variants evolved within the HLP lesion from intact EBNA-2 genes, while other variants appeared to be transmissible strains that directly infected the lesion. Intrastrain recombination within the HLP lesion also generated variation within the EBNA-2 polyproline region. Cloning and sequencing of HLP cDNA demonstrated transcription from the internally deleted EBNA-2 open reading frame, indicating that these variant genes are expressed in HLP. Comparative analysis of the HLP EBNA-2 sequences confirmed previous findings of EBV coinfection with multiple types and strains. Sequence variation of these wild-type genes demonstrated that EBNA-2A sequences distinguish at least two separate strains and a variety of substrains of EBV type 1. Two of the HLP EBNA-2A sequences contained amino acid changes in a cytotoxic T-cell epitope within an otherwise highly conserved region of the gene. These data indicate that EBV coinfection, strain variation, and recombination within the EBNA-2 gene are common features of HLP and suggest that the expression of internally deleted EBNA-2 variants could contribute to EBV pathogenesis in permissive infection.

Oral hairy leukoplakia (HLP) is an oral mucosal lesion characterized by the productive replication of the Epstein-Barr virus (EBV) within squamous epithelial cells of the lateral borders of the tongue, occurring most commonly in patients infected with the human immunodeficiency virus (15). Some HLP lesions are coinfected with both EBV type 1 (EBV-1) and EBV-2, on the basis of typing of the Epstein-Barr nuclear antigen 2 (EBNA-2) gene, and some HLP lesions may be coinfected with multiple strains of the same EBV type (39). In HLP, the productively replicating EBV recombines within and around the EBNA-2 gene, generating a heterogeneous group of viral variants that may be defective for EBNA-2 function (39). On the basis of analyses of sequential HLP biopsy specimens from the same patient, virus variants containing EBNA-2 gene rearrangements are capable of replicating efficiently and persisting within a lesion (39).

PCR amplification of the EBNA-2 coding sequence from HLP DNA generated products representing the full-length sequence, as well as a variety of smaller products suggestive of internally deleted forms of the gene (39). The heterogeneity of internally deleted EBNA-2 variants detected for both EBNA-2A (EBV-1) and EBNA-2B (EBV-2) in different HLP specimens suggested that they represented EBNA-2 recombination events within the lesion rather than superinfections with multiple independent strains. Their presence in a majority of the HLP specimens examined indicated that these variants occur frequently in HLP and may be important in productive EBV infection (39).

In this study, the EBNA-2 rearrangements were analyzed in six different HLP biopsy specimens by PCR amplification and sequencing. Several different patterns of genetic deletion were detected within both the polyproline and unique sequences of EBNA-2. Examination of EBNA-2 transcription indicated that internally deleted EBNA-2 genes are expressed in HLP lesions. Some of the variants evolved during viral replication within the HLP lesions, while others appeared to be independent strains. Sequence variation of the EBNA-2 gene defined at least two different strains of EBV-1 and confirmed that some HLP lesions are coinfected with multiple types, strains, or substrains of EBV.

MATERIALS AND METHODS

PCR amplification. HLP biopsy specimens were obtained from human immunodeficiency virus-seropositive volunteer patients, and the DNA was extracted as described elsewhere (12, 28). Single-stranded DNA oligonucleotides synthesized for use as PCR and sequencing primers are listed in Table 1. Primer sequences were designed from regions of the EBNA-2 gene common to both EBNA-2A and EBNA-2B and amplified both types of EBV DNA with equal efficiency. PCR amplifications of HLP DNA using oligonucleotides 1 and 8, and of HLP cDNA using oligonucleotides 9 and 8, were performed with 2 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass.); a 1:10 dilution of 10× reaction buffer as supplied with the Vent polymerase; 200 μM (each) dATP,

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TABLE 1. Synthetic oligonucleotides for PCR, sequencing, and probes

Oligonucleotides	Sequence (5′ to 3′)	Coordinates
1	CTCTCTAGACTATCTTGCGTTAC ATGGGGGACA	48515–48538
2	TTGTGTCCAGGCATCCCTGCGCT	48827–48805
3	AGGGATGCCTGGACACAA	48810–48827
4	TGGGGTGCTTTGATG	49307–49293
5	CATCAAAGCACCCCA	49293–49307
6	AGGCATGCTAGGACTGGA	49652–49635
7	TCCAGTCCTAGCATGCCT	49635–49652
8	CTCCTGCAGCGAGGTCTTTTACT GGGTCC	49952–49932
9	GCGCCAATCTGTCTACATAG	47940–47959
10	CCCCATGTAACGCAAGATAG	48534–48515
11	CGGGTGCTTAGAAGGTTGTT	48473–48454

dGTP, dCTP, and dTTP; and a 0.5 pM concentration of each primer in 50- or 100-μl reaction volumes. In some cases, 7-deaza-dGTP (Boehringer Mannheim, Indianapolis, Ind.) was substituted for dGTP in a ratio of 3:1 to increase amplification efficiency. Amplification proceeded for a total of 40 cycles with denaturation at 98°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. PCR amplifications of HLP cDNA using oligonucleotides 9 and 10 were performed with 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.); a 1:10 dilution of 10× reaction buffer as supplied with the *Taq* polymerase; 200 μM (each) dATP, dCTP, and dTTP; 50 μM dGTP; and 150 μM 7-deaza-dGTP (Boehringer Mannheim) with a 0.5 pM concentration of each primer in a 20-μl reaction volume. Amplification proceeded for a total of 40 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min.

RNA purification and cDNA synthesis. RNA was isolated from DNA by ultracentrifugation on a 4 M guanidine thiocyanate-cesium chloride step gradient as described previously (12). Residual DNA in the RNA was digested with DNase I (Boehringer Mannheim). After priming with oligo-dTTP, cDNA was synthesized from 20 μg of HLP RNA with Superscript reverse transcriptase (Gibco BRL, Grand Island, N.Y.) in the presence of 100 μM (each) dATP, dCTP, dGTP, and dTTP.

Probes and Southern blots. Restriction enzyme-digested HLP DNA and PCR products were subjected to agarose gel electrophoresis, transferred to a supported nitrocellulose membrane (Micron Separations Inc., Westboro, Mass.), and hybridized by Southern blot. The RNA probes designated 2A-YH and 2B-YH, which have been described elsewhere (39), were synthesized from cloned EBV DNA fragments using [α-^{32}P]UTP (Amersham, Arlington Heights, Ill.) and SP6 RNA polymerase (Promega). These probes identify both the *Bam*HI Y and H portions of the EBNA-2 gene and also differentiate between the EBNA-2A and EBNA-2B forms of the gene. RNA probe 2A-Y contains the EBV sequence from the *Hin*dIII site in *Bam*HI-Y (coordinate 48039) to the polyproline region of the EBNA-2A gene (coordinate 48737). RNA probe 2A-dH contains the sequence from the *Nco*I site in EBNA-2A (coordinate 49467) to the *Aha*III site in U2 (coordinate 50305) of the *Bam*HI H fragment. Probes 2A-Y and 2A-dH hybridize to EBNA-2 and surrounding sequences from the *Bam*HI Y and H fragments, respectively. These two probes were synthesized from cloned DNA fragments by using [α-^{32}P]UTP (Amersham) and T3 or T7 RNA polymerase (Promega). Single-stranded oligonucleotide 11 (Table 1) was labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (New England Biolabs) for use as an oligoprobe.

Cloning of PCR products. In some cases, the entire contents of a PCR mixture were cloned, while in other cases, specific bands were isolated by gel electrophoresis and purified with the GeneClean Kit (Bio 101, La Jolla, Calif.) before further amplification and cloning. *Xba*I and *Pst*I restriction enzyme sites within PCR primers 1 and 8, respectively, allowed efficient cloning into plasmid vector pGem3Z (Promega). PCR products generated from primers 9 and 8 were cloned with restriction enzymes *Sma*I and *Pst*I. Ligated PCR products and vectors were grown in *Escherichia coli* DH5αF′IQ, which is a *recA* mutant and deficient for in vitro recombination (Gibco BRL). Bacterial colonies containing the appropriate cloned PCR products were identified by in situ hybridization with probe 2A-YH or 2B-YH. Plasmid DNA for sequencing was extracted from bacterial cell cultures by alkaline lysis and was purified on Qiagen-tip columns (Qiagen Inc., Chatsworth, Calif.) or was isolated as described previously (36).

DNA sequencing. The DNA sequence was obtained from cloned DNA by the dideoxynucleotide chain termination method with [α-^{35}S]ATP (Amersham) and Sequenase (version 2.0) enzyme (United States Biochemical, Cleveland, Ohio). The sequencing reactions were primed with oligonucleotides matching the SP6 and T7 RNA polymerase promoter primer sequences within vector pGem3Z (Promega) as well as oligonucleotides 1 to 8 (Table 1). Sequencing reaction products were run on 8% denaturing polyacrylamide (Long Ranger; AT Biochem Inc., Malvern, Pa.) gels with 25% formamide.

RESULTS

DNA structure of EBNA-2 deletions. To determine the DNA structure of the EBNA-2 deletion variants detected in HLP (Fig. 1), HLP DNA from six different human immunodeficiency virus-infected patients (LC, PL, VL, WL, LH, and MT) was analyzed by PCR amplification, cloning, and sequencing of the EBNA-2 gene. PCR amplification of HLP DNA from patient LC demonstrated the presence of four different sizes of the EBNA-2A coding sequence: 1,360, 1,000, 750, and 420 bp (Fig. 1). EBV-2 represented by EBNA-2B was not detected. Three of the four products were cloned and sequenced (LC1360, LC746, and LC422) and demonstrated several different patterns of internal EBNA-2A deletion (Fig. 2A). All three clones contained internal deletions within the polyproline region as compared with the prototype EBNA-2A strain, B95-8 (2). Two of the clones, LC746 and LC422, each contained unique, larger deletions of EBNA-2 sequence to the right of the polyproline region.

Amplification of DNA from patient PL yielded product bands of 850, 510, and 420 bp of EBNA-2A but not of EBNA-2B (Fig. 1). A band representing the full-length, intact form of the gene was not detected. Three clones (PL512, PL503, and PL422) representing the two smaller product bands were obtained and sequenced (Fig. 2B). Similar to the LC clones, each PL clone contained a different sequence within the polyproline region, including both deletions and an insertion of sequence as compared with the B95-8 strain. Each of the three PL clones also contained an identical large deletion from within sequences to the right of the polyproline region. The structure of this deletion was identical to that of clone LC422.

Amplification of DNA from patient VL generated EBNA-2A PCR product bands of 1,450, 950, and 560 bp (Fig. 1). Clones of the 950- and 560-bp products (VL947 and

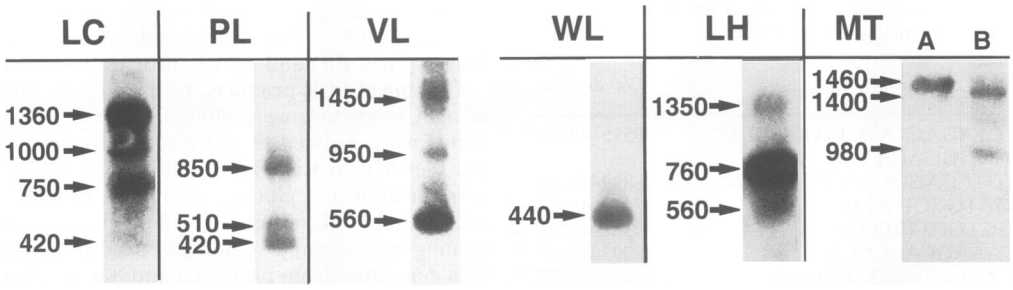

FIG. 1. PCR amplification of the EBNA-2 gene from HLP DNA. HLP DNA from six different patients was amplified by PCR using oligonucleotide primers 1 and 8 and hybridized to the 2A-YH probe (patients LC, PL, VL, WL, LH, and MT [MT lane A]) and the 2B-YH probe (MT only [lane B]). These probes differentiate EBV-1 from EBV-2 and identify sequences from both *Bam*HI-Y and -H. PCR product sizes are given in base pairs.

VL560) were obtained and sequenced (Fig. 2C). The two clones contained large internal EBNA-2A deletions that differed from each other and from those of LC and PL. In both VL947 and VL560, the deletions included the polyproline region and part of the amino-terminal sequence.

DNA from patient WL was amplified and demonstrated a 440-bp EBNA-2A product without evidence of a full-length, intact form of the gene (Fig. 1). Cloning and sequencing the PCR product (WL441) demonstrated another unique, large deletion that included the polyproline sequence (Fig. 2D). The deletion in this clone began at the same 5′ site as in clone VL560 but ended at a different 3′ site.

Amplification of DNA from patient LH produced 1,350-, 760-, and 560-bp EBNA-2A products (Fig. 1), but only the 760-bp band was cloned and sequenced (LH764) (Fig. 2E). This clone also contained a large deletion that included the polyproline region. It differed from the deletions described for the LC, PL, VL, and WL clones.

Amplification of DNA from patient MT yielded PCR product bands of 1,460 bp (EBNA-2A), 1,400 bp (EBNA-2B), and 980 bp (EBNA-2B) (Fig. 1). Clones representing the 1,460- and 980-bp products (MT1444 and MT982) were obtained and sequenced (Fig. 2F). Clone MT1444 contained full-length, intact EBNA-2A sequence, differing from B95-8 by both a deletion and a small insertion of sequence within the polyproline region. Clone MT982 was EBV-2 (EBNA-2B), and its polyproline region was identical to that of the laboratory Burkitt's lymphoma strain AG876 (9). Unlike the EBNA-2A clones, MT982 contained a unique, large deletion of sequence at the carboxy-terminal end of the gene.

These HLP EBNA-2 clones demonstrated a variety of deletion and insertion sites within the polyproline region (Table 2). In each clone, the EBNA-2 open reading frame was preserved by the sequence changes. Identical 5′ deletion start sites were detected in clones LC1360 and LC746 and in clones LC422, PL512, PL503, PL422, and MT1444. Identical 3′ deletion end sites were detected in clones PL512 and PL503 and in clones LC746, LC422, and PL422. Thus, identical polyproline deletions were detected in LC422 and PL422 and in PL512 and PL503. Clone PL512 differed from PL503 by the addition of one copy of the repeated sequence CCACCACCT between coordinates 48722 and 48759, while MT1444 also contained one additional copy of the repeated sequence CCC CCACCA between coordinates 48710 and 48756.

The HLP EBNA-2 clones also demonstrated a variety of deletion sites within the unique coding sequences (Table 3). For each of the EBNA-2A clones, the EBNA-2 open reading frame was disrupted by the large internal deletion, which introduced a new stop codon 3′ to the deletion. The EBNA-2B clone, MT982, preserved the EBNA-2 open reading frame after the deletion but also preserved the original stop codon immediately 3′ to the deletion. Identical large deletions were detected in clones LC422, PL512, PL503, and PL422. Clones VL560 and WL441 contained identical 5′ deletion start sites but different 3′ end sites. Aside from these identities, there was no apparent sequence homology between deletion start and end sites within each clone or among different clones (data not shown).

To verify that the variation among the HLP EBNA-2 clones represented the in vivo DNA structure of EBNA-2 rather than in vitro recombination during the PCR and cloning processes, EBNA-2A DNA from the B95-8 strain was examined after PCR amplification and cloning under identical conditions. A previous clone of B95-8 EBNA-2 that has been maintained in the laboratory for 10 years with repeated subcloning, transformation, and purification demonstrated complete sequence stability over that time (data not shown). DNA from that clone was amplified and cloned in a manner identical to that used for the HLP DNA. Eighteen clones were examined and contained the intact EBNA-2A coding sequence. In 17 of the 18 clones, there was no change in the sequence of the polyproline region, while 1 clone demonstrated an in-frame deletion of 90 bp. This deletion did not match any of the polyproline deletion sites of the HLP clones or the W91 EBV strain (Table 2) and may have arisen during the PCR amplification process (18). Given the low (6%) incidence of artifactual polyproline variation generated by the specific methods and conditions employed in this study, the polyproline regions described for the HLP EBNA-2 clones likely represent naturally occurring strain and variant differences.

To verify the origin of the larger deletions, HLP DNA was examined to detect and quantify the intact and deleted forms of the EBNA-2 gene present prior to PCR amplification. In the HLP clones LC746, VL947, VL560, WL441, and LH764, the EBNA-2 genes contained deletions that spanned the *Bam*HI restriction enzyme site between *Bam*HI-Y and -H. Therefore, two probes (2A-Y and 2A-dH) were designed to detect EBNA-2 and surrounding sequences from each side of the *Bam*HI-YH restriction site (Fig. 3). For patients LC, VL, WL, LH, and MT, HLP DNA was digested with *Bam*HI and hybridized with probes 2A-Y and 2A-dH (Fig. 3). The 2A-dH probe identified a typical 6.2-kb *Bam*HI H fragment in HLP specimens LC, VL, VL + 1 mo., and LH. However, in the specimens LC, VL, VL + 1 mo., WL, and LH, a 7.2-kb fragment was identified by both the 2A-Y and 2A-dH probes. The size and hybridization characteristics of the 7.2-kb frag-

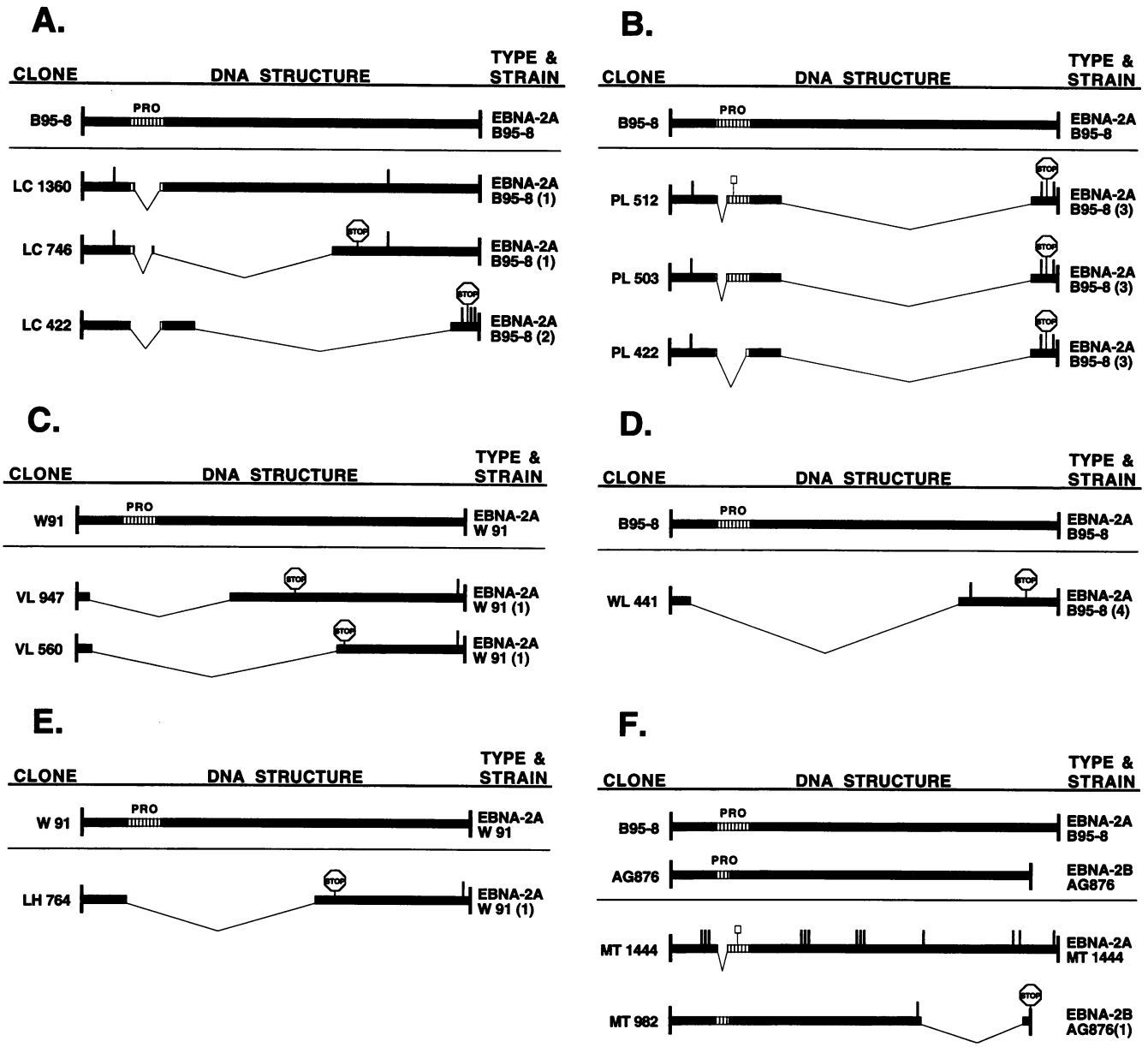

FIG. 2. Strain sequence and structural analysis of the HLP EBNA-2 PCR clones. Diagrams describe the EBNA-2 type, strain, and substrain and illustrate the structure of the EBNA-2 internal deletions. The DNA sequence and structure of the HLP clones are compared with those of the appropriate LCL reference strain, B95-8, W91, or AG876. Short vertical lines represent single-base-pair changes in sequence as compared with the reference strain above. Numbers in parentheses indicate substrain identity of each clone based on EBNA-2 sequence variation. PRO indicates the polyproline region of EBNA-2. Deletions are illustrated by thin lines. Insertions are illustrated by small rectangles. A stop sign indicates the location of the first stop codon after a deletion in the new reading frame.

ment were consistent with a fusion of the *Bam*HI Y (1.8-kb) and H (6.2-kb) fragments resulting from an approximately 0.5- to 1-kb deletion of EBNA-2 coding sequence spanning the *Bam*HI restriction site, as predicted by the HLP EBNA-2 clones.

Additionally, a second fragment larger than 7.2 kb in specimen WL also hybridized with both the 2A-Y and 2A-dH probes, suggesting the presence of EBNA-2 internal deletion as well as additional rearrangements in this fragment. For specimen LH, the 2A-dH probe identified a 6.2-kb *Bam*HI H fragment, but a corresponding 1.8-kb *Bam*HI Y fragment was not detected by the 2A-Y probe. This result suggests that additional EBNA-2 rearrangements have occurred in this HLP lesion beyond those represented by the 7.2-kb fragment. For the two specimens WL and MT + 5 mo., typical 1.8-kb *Bam*HI Y and 6.2-kb *Bam*HI H fragments were not detected, indicating that the 7.2-kb EBNA-2 deletion variants were the predominant forms of EBV replicating in these two HLP lesions. Remarkably, the 7.2-kb EBNA-2 deletion variant of patient VL persisted within the lesion over a 1-month period. In all HLP specimens, the quantity of the 7.2-kb EBNA-2 deletion variants exceeded 50 copies per cell. Thus, for these HLP specimens, EBV molecules consistent with both intact and internally deleted forms of the EBNA-2 gene were detectable

TABLE 2. EBNA-2 polyproline deletions

HLP clone or strain	5′ deletion start[a]	3′ deletion end[a]
LC1360	48696	48755
LC746	48696	48791
LC422	48687	48791
PL512[b]	48687	48710
PL503	48687	48710
PL422	48687	48791
MT1444[b]	48687	48707
W91 strain	48701	48715
B95-8 PCR control	48722	48802

[a] Coordinates are given according to the numbering of the genome of the B95-8 strain (2).
[b] The clone also contained an insertion of sequence into the EBNA-2 polyproline region (see text).

in abundance in the original HLP DNA before PCR amplification. Amplification of HLP DNA generated products that accurately detected the presence of the different forms of the EBNA-2 gene within the HLP lesion. Additionally, the size correlation between the PCR products and the HLP EBNA-2 clones indicated that the unique sequence deletions of these EBNA-2 genes did not arise during the cloning process.

EBNA-2 sequence variation and strain identification. Although the EBNA-2A gene (EBV-1) is highly conserved, the laboratory lymphoblastoid cell line (LCL) strain W91 is differentiated from LCL strain B95-8 by 13 nucleotide substitutions and a codon insertion (Table 4) (2, 7). With a majority of the changes occurring in the nonessential divergent region and with largely conserved amino acid sequences, B95-8 and W91 are functionally identical in lymphocyte transforming and transactivating assays (7). Previously, partially sequenced wild-type EBNA-2A genes from African and New Guinean isolates were found to closely resemble the W91 strain (1). In the present study, a large degree of sequence variation was detected in EBNA-2A genes cloned from HLP specimens; however, the sequences of most of the clones conformed to the sequence patterns of either the B95-8 strain or the W91 strain.

In Fig. 2, vertical lines above the horizontal bars represent the locations of single-base-pair changes in the EBNA-2 sequence relative to the reference strain above. Sequence variation within the polyproline region has been excluded from this analysis. All three LC clones contained an EBNA-2 sequence that differed from that of B95-8 by only 2 or 3 bp (Fig. 2A) but contained none or only one of the W91 signature

TABLE 3. EBNA-2 internal deletions of unique sequences

HLP clone	5′ deletion start[a]	3′ deletion end[a]
LC746	48764	49413
LC422	48922	49850
PL512	48922	49850
PL503	48922	49850
PL422	48922	49850
VL947	48559	49070
VL560	48577	49472
WL441	48577	49591
LH764	48677	49368
MT982	944	1318

[a] Coordinates are given according to the numbering of the genome of the B95-8 strain (2), except for those of HLP clone MT982, which are given as the nucleotide number of the EBNA-2B coding sequence (9).

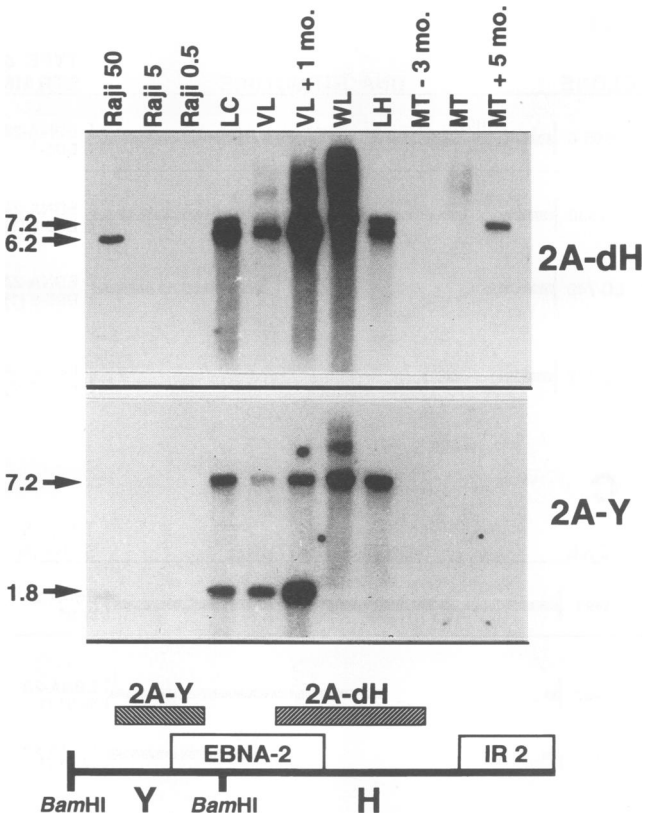

FIG. 3. EBNA-2 deletion variants in vivo. Eight samples of HLP DNA from five different patients (LC, VL at two sequential time points, WL, LH, and MT at three sequential time points) were digested with *Bam*HI and hybridized in duplicate with probes 2A-Y and 2A-dH, whose hybridization domains are shown by the hatched bars above the schematic diagram of the EBNA-2 gene region. Reconstructions of Raji EBV LCL DNA indicate the EBV genome copy number per cell. Fragment sizes are indicated in kilobases.

changes. Therefore, the LC clones represented substrains of the B95-8 strain. Clone LC746 contained a sequence in its undeleted regions that was identical to that in LC1360, but both differed from the clone LC422 sequence by 4 bp in the small amount of undeleted sequence common to all three. The sequence identity between LC1360 and LC746 indicated substrain identity between these two clones and implied an evolutionary process within the lesion, generating the large deletion of LC746 from an intact form of the gene represented by LC1360. Similarly, the variation between the polyproline regions of these two clones indicated a second site of intrastrain viral recombination. The sequence variation of the LC422 clone identified it as a separate B95-8 substrain, and its presence in the HLP lesion implied coinfection of the HLP lesion with two different B95-8 substrains of the same EBV type, as suggested for HLP by previous studies (39).

All three PL clones contained identical sequences in their undeleted regions that differed only slightly from that of B95-8 (Fig. 2B), with no W91 signature changes, indicating a B95-8 substrain identity between them. As demonstrated by the LC clones, intrastrain recombination within the polyproline region generated these three PL variants. Remarkably, the large deletion was identical in all three PL clones and was identical to that of clone LC422, while the EBNA-2 sequence between the PL clones and LC422 differed by only 2 bp. The similarities

TABLE 4. EBNA-2A strain signatures of W91 and MT1444 compared with those of B95-8

B95-8 coordinate[a]	EBNA-2 codon	Nucleotide change	Amino acid change	Conserved or similar amino acid	EBNA-2 functional domain	Strain W91	Strain MT1444
48619	39	A→C	Gln→Pro	−	Amino terminal	−	+
48628	42	A→G	Asp→Gly	−	Amino terminal	−	+
48629	42	C→A	Asp→Gly	−	Amino terminal	−	+
48990	163	A→G	Arg→Glu	−	Divergent	+	+
48991	163	G→A	Arg→Glu	−	Divergent	−	+
48991	163	G→T	Arg→Val	−	Divergent	+	−
48998	165	C→A	Val→Val	+	Divergent	+	+
49057	185	A→G	Gln→Arg	−	Divergent	+	−
49091	196	G→T	Met→Ile	+	Divergent	+	−
49113	204	A→T	Thr→Ser	+	Divergent	+	−
49136/49137	211/212	—[b]→CTC	— →Leu	−	Divergent	+	−
49170	223	C→T	Leu→Leu	+	Divergent	+	−
49211	236	C→T	Thr→Thr	+	Divergent	−	+
49218	239	C→A	Gln→Thr	−	Divergent	−	+
49219	239	A→C	Gln→Thr	−	Divergent	−	+
49444	314	T→C	Leu→Ser	−	Protein binding	−	+
49449	316	C→A	His→Asn	−	Protein binding	+	−
49613	370	T→A	Pro→Pro	+	Nonessential	+	−
49754	417	G→A	Thr→Thr	+	Nonessential	+	−
49777	425	T→C	Ile→Thr	−	Acidic	−	+
49793	430	C→T	Ser→Ser	+	Acidic	−	+
49913	470	A→T	Ser→Ser	+	Acidic	+	−
49923	474	T→C	Tyr→Asn	−	Acidic	+	−
49930	476	A→G	Glu→Gly	−	Nuclear localization	+	+

[a] Coordinates listed in this analysis exclude the EBNA-2 polyproline region and EBNA-2 sequences contained within the oligonucleotide PCR primers 1 and 8.
[b] —, none.

among these clones from different patients suggested either that this specific deletion has evolved independently in two different HLP lesions or that variant strains or substrains containing this specific deletion and circulating in the community have independently infected these two HLP lesions. The absence of a full-length form of EBNA-2 by PCR in the PL HLP lesion could suggest infection by this deletion variant rather than its evolution within the lesion. Regardless of its origins, the existence of this specific deletion variant in more than one HLP suggested that it possesses biological advantages for permissive EBV infection in HLP.

Both VL947 and VL560 contained identical sequences in the undeleted regions. VL947 contained the signature W91 codon insertion, and both clones differed from W91 by only a single nucleotide change (Fig. 2C), indicating W91 substrain identity of VL947 and VL560. Similar to LC746, both VL clones were therefore derived from a full-length, parental form of the EBNA-2A gene, likely represented by the uncloned 1,450-bp PCR product.

Clone WL441 contained a large deletion encompassing the most divergent sequence between W91 and B95-8. The sequence of WL441 differed from that of W91 by 4 nucleotides but differed from that of B95-8 by only 1 nucleotide (Fig. 2D). Thus, clone WL441 likely represented a B95-8 substrain, separate from the substrains of the LC and PL clones. Analogous to the PL HLP lesion, no full-length form of EBNA-2 was detected by PCR in the WL HLP lesion, again suggesting the possibility of direct infection by this EBNA-2-deleted viral variant.

Although the region of the W91 signature codon insertion was deleted in this clone, LH764 contained a sequence in the undeleted regions that closely matched that of W91 (Fig. 2E) and that matched identically with the undeleted sequences of the VL clones. Therefore, VL947, VL560, and LH764 may represent the same W91 substrain independently infecting the HLP lesions of two patients. Differences in the deletion sites among these three clones again indicated that deletion site variation was not sequence specific.

Clone MT982 contained a sequence in its undeleted region that was nearly identical to the EBNA-2B sequence of the laboratory Burkitt's lymphoma strain AG876 (Fig. 2F). The concurrent presence of the EBNA-2A gene within this HLP lesion, MT1444, confirmed at the sequence level that some HLP lesions are coinfected with both EBV-1 and EBV-2 (39). The sequence of MT1444 differed from that of B95-8 by 13 nucleotide substitutions (Fig. 2F). Although three of those changes matched W91 sequence changes, MT1444 lacked the signature codon insertion and differed from W91 by 20 nucleotide substitutions. The nucleotide and amino acid substitutions of MT1444 are compared with the sequences of both B95-8 and W91 in Table 4. With significant sequence divergence from both B95-8 and W91, the EBNA-2A sequence of MT1444 likely represents a new, third strain of EBV-1. On the basis of the known functional domains of the EBNA-2A protein (6, 7, 23, 24), the changes in MT1444 would likely not alter transformation and transactivation functions of this gene in B lymphocytes.

EBNA-2 transcription in HLP. For each of the Epstein-Barr nuclear antigens 1 to 6, expression requires unique differential splicing of a single mRNA transcript before translation (31). The RNAs of 10 different HLP biopsy specimens from six different human immunodeficiency virus-infected patients were reverse transcribed into cDNA and analyzed by PCR amplification for EBNA-2 specific posttranscriptional processing. In this assay, PCR primers 9 and 10 flanked the intron between the Y_2 and the Y_H exons (Fig. 4) (31) such that amplification of the correctly processed EBNA-2 message generated a 209-bp product. Unspliced mRNA or contaminating DNA was detected by a 595-bp PCR product. The 209-bp PCR product was detected in 6 of 10 HLP specimens from four of the six patients, including patients VL and WL (Fig. 4). These results indicated that the appropriate transcriptional

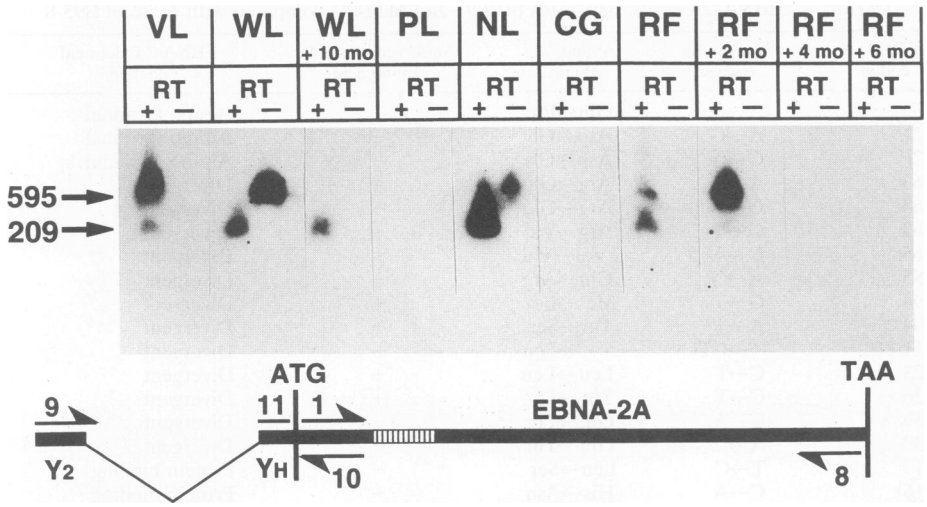

FIG. 4. Expression of the EBNA-2 open reading frame in HLP. Ten samples of HLP cDNA from six different patients (VL, WL at two sequential time points, PL, NL, CG, and RF at four sequential time points) were amplified by PCR using oligonucleotide primers 9 and 10. These primers amplify across the intron between the Y_2 and the Y_H exons upstream of the EBNA-2 coding sequence. The PCR products were identified by hybridization to oligonucleotide probe 11. The 209-bp products represent the correct mRNA splice for EBNA-2 expression, while the 595-bp products represent unspliced mRNA or contaminating DNA. RT, reverse transcriptase.

processing for the expression of the EBNA-2 open reading frame occurs and persists in some HLP lesions.

To confirm the presence of the Y_2-to-Y_H splice and determine if the internally deleted forms of EBNA-2 may be expressed in HLP, cDNA from patient WL was PCR amplified with primers 9 and 8, which span both the Y_2-to-Y_H splice site and the EBNA-2 open reading frame (Fig. 4). Cloning and sequencing this PCR product from WL cDNA demonstrated the presence of the Y_2-to-Y_H splice in conjunction with the internal deletion of the EBNA-2 coding sequence (Fig. 5). The internal EBNA-2 deletion and the undeleted EBNA-2 sequences of the WL cDNA clone matched identically with those of the WL DNA clone WL441, indicating that the internally deleted EBNA-2 genes of HLP lesions are transcribed and appropriately processed to be translated into protein within an HLP lesion.

DISCUSSION

Previous studies have demonstrated that EBV exists in HLP as a continuously evolving, heterogeneous population of viral types, strains, and recombinant variants (39). EBV-1 and EBV-2 are distinguished by nearly 40% sequence divergence of the EBNA-2 gene (9) and up to 20% divergence in the EBNA-3, EBNA-4, and EBNA-6 genes (32). In the laboratory, EBV strains have been defined by restriction fragment length polymorphisms arising from sequence variation at restriction sites and/or variation of repetitive sequences within the fragment (3, 8, 16). Previous and present data demonstrate that EBV intrastrain recombination within both repetitive and unique sequences of the EBNA-2 and latent membrane protein 1 genes generates variants during productive replication in HLP (27, 39). Thus, in the study of permissive EBV infections, EBV strains could be more accurately distinguished by sequence identity or divergence within genes that are known to differ between isolates (7, 27).

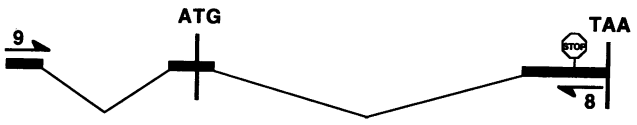

FIG. 5. Sequence and structural analysis of an HLP cDNA. The PCR amplification product of oligonucleotide primers 9 and 8 from patient WL HLP cDNA was cloned and sequenced. It contained the mRNA splice from exon Y_2 to exon Y_H continuously joined to EBNA-2 coding sequence that identically matched the sequence and structure of the HLP DNA clone WL441.

The present study validates and extends this definition of an EBV strain by demonstrating that most wild-type EBNA-2A nucleotide sequences assort with known strain variation from laboratory LCLs. The one HLP EBNA-2 clone not resembling either B95-8 or W91 likely represents a third wild-type strain, although confirmation will require examination of more EBV isolates. EBV strains may be further divided into substrains on the basis of additional nucleotide substitutions within a given EBNA-2A strain sequence. In the HLP of patients LC, PL, and VL, this definition of EBNA-2A substrain enabled the differentiation between EBV coinfection and the generation of deletion variants through recombination. The distinction between EBNA-2A strain and substrain is clearly one of degree and dependent on the rate of evolutionary changes in vivo (EBV polymerase errors), the rate of mutation in vitro (*E. coli* polymerase errors), and the fidelity of the thermostabile DNA polymerase used for PCR amplification. On the basis of the sequence stability of the EBNA-2 clone of B95-8, the contribution of *E. coli* polymerase to variation appears negligible. The Vent DNA polymerase used in this study contains a 3′-to-5′ proofreading exonuclease activity which results in very high-fidelity nucleotide incorporation. Therefore, PCR amplification is also unlikely to be the source of sequence variation.

The biological implications of strain and substrain sequence variation are unclear, but some amino acid substitutions may play a role in antigenic variation of a critical viral protein, resulting in evasion of host immune recognition. In persons expressing HLA-A11 major histocompatibility complex class I molecules, the HLA-A11 restricted cytotoxic T-lymphocyte response is to a specific epitope of the EBNA-4 protein (11). In

human populations with a high prevalence of HLA-A11, the predominant EBV-1 strain has mutated a single amino acid residue within that epitope, abrogating the host cytotoxic T-lymphocyte recognition of that protein (10). For EBNA-2A, two cytotoxic T-lymphocyte epitopes have been defined: amino acids 42 to 51, associated with HLA-A2, and amino acids 276 to 291, associated with HLA-B18 (21). The EBNA-2A substrain represented by clones LC1360 and LC746 has mutated amino acid 42 (Asp→Asn) within the first epitope. Similarly, the EBNA-2A strain represented by clone MT1444 has mutated the same amino acid (Asp→Gly) within the first epitope. Both of these amino acid changes result in significant side chain substitutions that may alter the immune processing and recognition of that epitope in persons expressing HLA-A2 and possibly other HLA types.

The G+C-rich, repetitive EBNA-2 polyproline region is dispensable for transactivation and transformation function in B lymphocytes (7) and varies in length among strains, especially between EBV-1 and EBV-2 (7, 9). Even two clones of the same LCL strain (B95-8) from two different laboratories have evolved a 9-nucleotide difference in polyproline length (2, 9). Analogous to the variable Gly-Ala amino acid repeats of the EBNA-1 gene (17), polyproline variation could account for some of the variation in EBNA-2 protein size detected among wild-type EBV isolates (13, 14, 34). While homologous recombination is a consistent feature of productive EBV replication both in vitro and in vivo (27, 38, 39), examples from herpes simplex virus type 1 suggest that recombination also occurs at specific G+C-rich recognition sites denoted by specific DNA conformations (19, 40). Thus, viral recombination within the EBNA-2 polyproline region could account for the polyproline variations detected in the same-strain EBNA-2 genes from a single HLP specimen.

During productive replication both in vitro and in vivo, EBV recombination within nonrepetitive genome regions generates viral variants through the deletion, insertion, or juxtaposition of sequences (4, 20, 28, 29, 35). The heterogeneous DNA of the P3HR-1 LCL replicates independently and is transmissible in culture (25), while EBNA-2-deleted variants similar to P3HR-1 are detectable in oropharyngeal secretions from 15% of healthy adults and in over 90% of HLP specimens (35, 39). Present data from HLP indicate that EBNA-2 internal deletion variants not only arise through recombination within the HLP lesion but may exist as independent strains as well. Given the abundance and variety of deletions detected, EBNA-2 appears to be an exceptionally active region for viral recombination during productive replication. However, the diversity of sites utilized in EBNA-2 recombination reveals neither homology nor sequence specificity nor consistently high G+C content to suggest a single mechanism of recombination.

Previous studies have not convincingly demonstrated the expression of the EBNA-2 gene in HLP. EBNA-2 transcripts were not detected by Northern blotting of HLP RNA, nor were they detected in two cDNA libraries of HLP biopsy specimens (22). The presence of EBNA-2 protein in HLP histologic tissue sections was reportedly detected in approximately 50% of specimens by using monoclonal antibody PE2 (33, 37). In the present study, PCR analysis of HLP cDNA detected EBNA-2 transcripts in approximately two-thirds of the HLP specimens, including transcription of internally deleted EBNA-2 genes. Translation of deleted EBNA-2 gene transcripts would produce proteins lacking the PE2 epitope (7) and probably lacking many human antibody epitopes, making the putative variant proteins difficult to detect by immunologic techniques.

The EBNA-2 gene product is required for EBV transformation of B lymphocytes, differing in its transformation efficiency between EBNA-2A and EBNA-2B (5). An important regulatory gene in B-lymphocyte infection, EBNA-2 transactivates a number of cellular and viral genes and coordinates the expression of other EBNA genes (7, 30). Like P3HR-1, EBV strains deleted for the EBNA-2 gene are transformation incompetent but can superinfect latent LCLs and replicate efficiently (26, 29, 35). However, the role for EBNA-2 in epithelial infection is uncertain. Latently infected epithelial cells of nasopharyngeal carcinoma do not express the EBNA-2 gene (41), and EBNA-2 is not required for productive replication in mucosal epithelial infections (35).

The abundance and frequency with which EBNA-2-defective variants occur and persist in HLP suggest positive selection for the biological properties of these variants (35, 39). The domains for nuclear localization, protein-protein interactions, and DNA transcriptional transactivation within the carboxy terminus of the protein have been deleted from all of the EBNA-2 variants detected in these HLP lesions (6, 7, 23, 24). This loss of EBNA-2 function could facilitate the initiation of viral replication by failing to stimulate latency-associated gene expression, an advantageous attribute in a permissive environment. The diversity of internally deleted EBNA-2 genes may simply reflect positive selection for a variety of random viral variants that are incapable of expressing a functional EBNA-2 protein.

Alternatively, the expression of the internally deleted EBNA-2 gene could play an as yet undefined role in permissive epithelial infection. These genes have preserved some or all of the amino-terminal domain which is 98% conserved at the nucleotide level among the HLP EBNA-2A strains and 80% conserved at the amino acid level between EBNA-2A and EBNA-2B (9). The maintenance and expression of the EBNA-2 amino-terminal domain in HLP may indicate viral conservation of a functionally important domain for permissive infection of mucosal epithelium. The prevalence and persistence in HLP of the internally deleted EBNA-2 variants suggest that the role of EBNA-2 recombination in permissive EBV infection warrants further study.

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